

Roles of IL-4 and other factors in trichosanthin-induced ovalbumin-specific IgE response¹

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XU Wei-Feng, JI Yong-Yong, WU Ya-Di, LIN Guo-Mei, YE Min, SUN Bing²

(The Laboratory of Molecular Immunology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China)

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be involved in, but CD40L may play a more important role.

ABSTRACT

AIM: To study the mechanism of trichosanthin (TCS)-induced ovalbumin (OVA)-specific immunoglobulin E (IgE) response *in vivo*. **METHODS:** To determine whether interleukin-4 (IL-4) was involved in TCS-induced IgE production, TCS and OVA co-immunized mice were treated with anti-IL-4 monoclonal antibodies (mAb) and OVA-specific serum IgE production was measured by enzyme-linked immunosorbent assay (ELISA). To distinguish whether recombinant IL-4 (rIL-4) was sufficient to support OVA-specific IgE response, OVA alone immunized mice were treated with rIL-4 and OVA-induced IgE production were examined by ELISA in the serum. To determine whether additional factors were involved in TCS-induced IgE response, the kinetic expression of CD40 ligand (CD40L), tumor necrosis factor- α (TNF- α), and interleukin-13 (IL-13) were measured by semi-quantitative RT-PCR in draining mesenteric lymph node (MLN) from TCS-immunized mice. **RESULTS:** TCS-induced OVA-specific IgE production was suppressed by anti-IL-4 antibody, whereas IL-4 alone could not induce OVA-specific IgE production. CD40L, TNF- α , and IL-13 all expressed high levels in MLN after both primary and secondary immune responses. Among them CD40L had the similar transient expression peak to that of IL-4. **CONCLUSION:** IL-4 was indispensable for TCS-induced OVA-specific IgE production, and the other three factors examined may also

INTRODUCTION

Trichosanthin (TCS), the major effective component from Chinese herb *Trichosanthes Kirilowii* Maxim, with abortion induction effect, is also effective in anti-cancer and anti-HIV therapy^[1-7]. Since TCS is a potent allergen, thus limits its therapeutic use in clinic. Immunoglobulin E (IgE) is the principle mediator in allergy. In our IgE response mouse model, TCS was able to induce TCS-specific IgE response without any adjuvant^[8]. In addition, TCS co-immunization with ovalbumin (OVA) was able to help OVA to induce OVA-specific IgE response^[9], while OVA alone immunization could not. However, this help could happen only when TCS was injected one day earlier than OVA. So there must be some non-specific and transiently expressed factors involved in this process. To synthesize IgE, B cells require some important signals during T-B cooperation^[10,11]. One signal is interleukin-4 (IL-4), which initiates the activation of transcription of constant region of ϵ (C ϵ) gene^[10]. Our previous work showed that after TCS immunization the expression of IL-4 mRNA in mesenteric lymph node (MLN) was quick and transient and could be fitted well into the "narrow window" help of TCS to OVA^[12]. So we supposed that IL-4 might be one of the transiently expressed critical factors in IgE response. In the current work, we used neutralizing anti-IL-4 monoclonal antibody and recombinant IL-4 to proof this hypothesis.

CD40L, mainly expressed on activated T cells, can cognate with CD40 on B cells and exert great impact on B cell proliferation and differentiation. It has been shown that CD40/CD40L interaction is the second critical signal for IgE production^[13-15]. Membrane TNF- α is required for antibody response^[16] and facilitates the induction of surface molecules on B cells that are important in T-B co-

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² Correspondence to Prof SUN Bing.

Phn 86-21-6471-1340. Fax 86-21-6433-1090.

E-mail bingsun@sum.sjnc.ac.cn

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operation^[17]. Since it has significant structural homology to CD40L, TNF- α may have some overlapping function with that of CD40L^[18]. IL-13 is a recently explored cytokine that shares one of receptor subunits with IL-4 thus shares many functions with IL-4^[19]. So, in consideration for other factors that may be involved in this TCS help for OVA, we also used the semi-quantitative RT-PCR to analyze the kinetic expression of CD40 ligand (CD40L), interleukin-13 (IL-13), and tumor necrosis factor- α (TNF- α) during the TCS-induced IgE response.

MATERIALS AND METHODS

Mice C57BL/6J, female, 9 - 12 weeks old, were obtained from the Animal Center of Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Grade II, Shanghai Animal Certificate No 153) and animals were bred according to the Institutional guidelines.

Antigen and reagents Crystallized TCS in trichosanthin injection solution (1.2 g/L) produced by Jin-San Pharmaceutical Factory (Shanghai, China). Anti-IL-4 monoclonal antibody (11B.11) was purchased from NCI-FCRDC (Frederick, MD, USA). Murine recombinant IL-4 was purchased from Schering Plough Research Institute (Kenilworth, UK) with specific activity 1×10^{11} U/g.

Treatment of animals with 11B.11 and rIL-4

Group I: negative control, OVA immunized alone on d +1. **Group II**: positive control. On d 0 mice were immunized with TCS (5 μ g, ip) and on d +1 the mice were immunized with OVA (10 μ g, ip). **Group III**: anti IL-4 mAb treated group. The mice were treated with 11B.11 (0.5 mg/d per mouse) at d -1, 0, and +1 and immunized the same as group II. **Group IV**: rIL-4 treated mice. The mice were treated with rIL-4 (105 U/d per mouse) on d 0, +1, and +2 and immunized with OVA on d +1. On d 21 all the mice were boosted with the same antigen as the first time in each group. Serum were collected on d 31 following immunization and OVA-specific IgE was determined by ELISA. Each group included 3 mice in two independent experiments.

ELISA The level of OVA-specific IgE antibody in serum was evaluated by ELISA as previously described^[9]. Briefly, 96-well plates (Corning Costar, Corning, USA) were coated with OVA (2 mg/L). After blocking the plates with bovine serum albumin (Sino-American Biotechnology Company, Shanghai, China)

and overnight incubation with serum samples, the plates were developed with horseradish peroxidase-conjugated goat anti-mouse-IgE subclass-specific antibodies (from Jingmei Biotech Co Ltd, Shanghai, China). The Absorbance at 450 nm was then measured with MICROPLATE AUTOREADER (BIO-TEK INSTRUMENTS INC, EL311, Vermont, USA).

Preparation of total cellular RNA from mesenteric lymph node of TCS-immunized mice

TCS was diluted in PBS and adjusted to 10 mg/L. Each mouse was injected ip with 0.5 mL of antigen solution. On d 30 after the primary immunization, another 0.5 mL TCS solution was injected into each mouse as previously described^[12]. The MLN of three mice were collected as a group on d 1, 2, 3, 5, 7, 11, 30, 31, 32, 33, 35, 37, 41, and 60 following immunization. The total RNA was directly extracted from draining MLN without culture. The experiment was repeated and the kinetic graphs of expression genes were drawn on the average value of these two experiments.

RT-PCR and quantitative analysis Total RNA was extracted from MLN with GIBCO-BRL TRIZOL Reagent (Rockville, USA) as directed by manufacturer. cDNA was synthesized using GIBCO-BRL M-MLV: 10 μ L of $5 \times$ first strand buffer, DTT 0.01 mol/L, 1 mmol/L of each dNTP, random hexamer primers 5 μ mol/L and RNA 20 mg/L in a total volume of 48 μ L. The mixture was denatured at 70 $^{\circ}$ C for 10 min then chilled on ice for 2 min. After adding reverse transcriptase M-MLV 400 U, the mixture was put in 37 $^{\circ}$ C water bath for 1 h and then inactivated at 95 $^{\circ}$ C for 5 min. The method was described in our previous work^[12]. Semi-quantitative PCR was based on Chelly's protocol^[20] with some modifications. PCR amplification was performed on CEL-BIO Temperature Cycling System (HYBAID OmniGene, Middlesex, United Kingdom). PCR system: 12 μ L of $10 \times$ reaction buffer, each dNTP 0.25 mmol/L, 0.75 pmol each sense and antisense primers, and 6 μ L of cDNA. Added double-distilled H₂O to 117 μ L. The mixture was denatured at 95 $^{\circ}$ C for 5 min, then 3 μ L of Taq DNA polymerase (3 U per μ L, Sino-American Biotechnology Company, China) was added as a "hot start" method to eliminate non-specific annealing. PCR was performed as following conditions: denaturation for 45 s at 94 $^{\circ}$ C, annealing for 45 s at 59 $^{\circ}$ C for CD40L and 61 $^{\circ}$ C for IL-13 and TNF- α , and extension for 45 s at 72 $^{\circ}$ C. Initially, target genes were amplified for 7 - 13 cycles and then the primers for internal standard, GAPDH, were added by the "primer-dropping" method. After another 14 - 17

cycles, 17 μ L of template of PCR reaction was collected from the following different cycles and templates were then separated by electrophoresis through 6 % polyacrylamide gels, stained with ethidium bromide and photographed. The density of target bands were measured by FURI Smartview-2000 (Shanghai FURI Science and Technology Co, Ltd). For each gene product, the optimal numbers of PCR cycles were determined. Thus the ratios of target over internal standard from 8 serial points of PCR reactions were analyzed and linear standard curve was made. The target mRNA expression was calculated from the curve. The sequences of primers and expected sizes of products are: GAPDH: Sense primer (SP): 5'-ACGACCCCTTCATTGACC-3'; Antisense primer (AS): 5'-AGACACCAGTAGACTCCACG-3' (194 bp). CD40L: (SP): 5'-TUGTGAAGAGATGAGAAGGC-3'; (AS): 5'-CCGATTAGAGCAGAAGGTG-3' (291 bp). IL-13: (SP): 5'-GGCTCTGGGCTTCATGGCG-3'; (AS): 5'-GCTGGAGACCGTAGTGGG-3' (484 bp). TNF- α : (SP): 5'-ACAAGCCGTAGCCACG-3'; (AS): 5'-TCCAAAGTAGACCTGCCC-3' (428 bp).

RESULTS

IL-4 was required to induce IgE production, but IL-4 alone could not induce OVA-specific IgE response To evaluate whether IL-4 production is involved in TCS induced OVA-specific IgE response, the TCS and OVA were combined to immunize mice. In this system, immunization with OVA alone failed to induce OVA-specific IgE production, however, when animals were treated with single injection of TCS one day before OVA immunization, the immunized mice were able to produce OVA-specific IgE^[9]. When anti-IL-4 mAb was used to block the endogenous IL-4 on d -1, 0 and +2 successively following TCS immunization, OVA-specific IgE production was completely inhibited (Fig 1). When animals were injected rIL-4 to replace the TCS "helping" on d 0, +1, and +2 following OVA alone immunization, OVA-specific IgE production failed to restore. These data suggested that in TCS induced OVA-specific IgE response, in addition to IL-4, other factors also played important roles.

CD40L mRNA expression showed similar pattern to IL-4 expression in MLN from TCS immunized mice CD40L expression was also transient; it peaked on d 3 and faded to nearly baseline level on d 5 both after the primary and secondary TCS immunization, and the first peak was higher than that of the secondary one (Fig 2). The results showed that CD40L expression

followed IL-4 mRNA expression and had a similar transient expression pattern to IL-4.

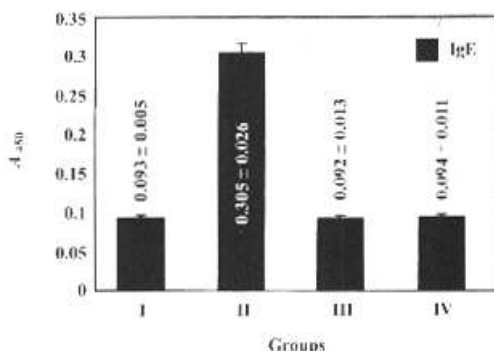


Fig 1. OVA-specific IgE production in rIL-4 and anti-IL-4 treated mice. $n = 3$. $\bar{x} \pm s$. Group I: negative control. Group II: positive control. Group III: anti-IL-4 mAb treated mice. Group IV: rIL-4 treated mice.

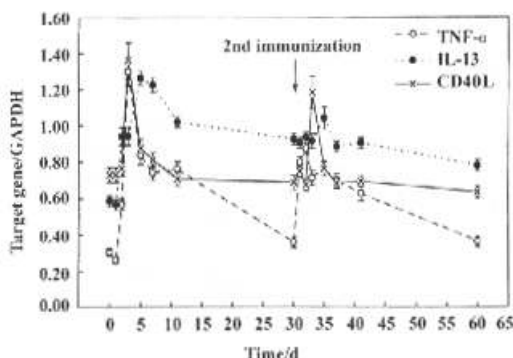


Fig 2. Kinetic expression of CD40L, TNF- α , and IL-13 genes. Expression of CD40L, TNF- α ; and IL-13 genes in MLN from TCS immunized mice. PCR products were electrophoresed through 6 % polyacrylamide gels as described in methods and materials. The ratio of intensity of target genes over GAPDH was used to express the level of mRNA expression. High ratio indicated high expression of target genes.

TNF- α and IL-13 mRNA expression showed different patterns to that of IL-4 and CD40L TNF- α 's first peak was nearly identical to that of CD40L, it peaked on d 3 but faded more slowly; on d 11 its level was still about half of the peak value (Fig 2). In contrast, IL-13 peaked on d 5, 4 d later than that of IL-4, and maintained at relatively high levels through all the rest time of experiment (on d 30 its expression level was still about 70 % of the peak on d 5. On d 30 and 60 its level was still significantly higher than its baseline level;

0.922 and 0.777 in comparison with 0.6 (Fig 2).

DISCUSSION

It has been accepted that IL-4 and CD40/CD40L interaction are two critical signals for IgE production^[10,11]. In our mouse model, we found TCS could help OVA to induce OVA-specific IgE response. However, this help could happen only when TCS was injected one day before OVA immunization. This means the factors that realizing this help must be expressed transiently. We supposed the transiently expressed genes during the IgE response were most likely the candidates for the help effects of TCS to OVA. For this reason, we analyzed the kinetic gene expression in TCS immunized mice. The expression of IL-4 in MLN after TCS injection was really transient, it reached peak on d 1 and decreased to base level quickly^[12]. In this work, we firstly used anti-IL-4 mAb to block IL-4 and found that OVA-specific IgE product was inhibited completely. Thus, it is likely that IL-4 is essential not only to TCS-induced IgE response, but also to IgE response of other unrelated antigen immunized shortly after TCS immunization. Next we asked whether rIL-4 was able to replace TCS and help OVA to induce IgE response. The data showed that supplement of exogenous rIL-4 failed to induce OVA-specific IgE production, suggesting that in addition to IL-4 other factors were also involved in IgE induction. So we further analyzed the kinetic expression of the other three genes (CD40L, TNF- α , and IL-13) that have a potential role in the induction of IgE response. The results showed all three factors had enhanced expression after first and second TCS immunization, suggesting that they play roles in the IgE response.

CD40L had the similar transient expression pattern to that of IL-4, implying its key role in the process of TCS induced OVA-specific IgE response. In fact, CD40L plays a crucial role in initiating the B cell response and is a key factor to induce the IgE switch recombination^[13,14]. So it seems that CD40L on TCS-specific T cells could bind directly to the CD40 on the OVA-specific B cells and stimulate these B cells to produce IgE.

Both membrane TNF- α (mTNF- α) and CD40L belong to the TNF superfamily. Based on their significant structural homology, it makes a sense to ask whether mTNF- α could act as a costimulator and induce IgE response in B cell in the similar way as CD40L did^[16]. In our experiments, the expression of TNF- α reached peak on day 3 after immunization and faded slowly. On d 11

after immunization, its level was still about half of the peak value. This sustaining high expression of TNF- α implied its role in the process. In fact, mAb against mTNF- α could strongly inhibit IgE synthesis^[21].

IL-13 shares many biological activities with IL-4. This is due to the fact that IL-13 and IL-4 receptor complexes share the same α -chain, which is important for their signal transduction. In fact, IL-13 is required for optimal induction of IgE synthesis^[19]. In our work, in contrast to IL-4 and CD40L, the expression of IL-13 was slower to reach the peak, but it was kept at relatively high levels during the whole process. This suggested that IL-13 played a more important role in the sustaining of the IgE response, rather than initiating it.

In conclusion, IL-4 was indispensable in TCS induced OVA-specific IgE production, but some other factors such as CD40L may be also involved.

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白介素-4 和其他因子在天花粉蛋白诱导的卵清白蛋白特异性 IgE 应答过程中的作用

徐卫锋, 季永镛, 吴亚娣, 林国妹, 叶 敏, 孙 兵² (中国科学院上海生命科学研究院生化细胞所分子免疫组, 上海 200031, 中国)

关键词 天花粉素; 免疫球蛋白类; 白介素-4; 白介素-13; 肿瘤坏死因子; 配体

目的: 研究小鼠模型中天花粉蛋白(TCS)诱导的卵清白蛋白(OVA)特异性的 E 型免疫球蛋白(IgE)应答反应的可能机理. **方法:** 首先用抗白介素-4(IL-4)的单抗治疗 TCS 和卵清白蛋白(OVA)免疫的小鼠, 通过减少内源性 IL-4 的水平, 以观察其对血清中 IgE 抗体水平的影响. 其次, 用重组 IL-4 处理小鼠, 通过增加外源性 IL-4 的水平观察其对血清中 OVA 特异性 IgE 形成的影响. 最后, 我们在 TCS 免疫小鼠 IgE 形成过程中, 用半定量 PCR 方法检测了腹腔淋巴结中 CD40 的配体(CD40L), 肿瘤坏死因子- α (TNF- α)和白介素-13(IL-13)基因表达的趋势. **结果:** 抗 IL-4 的单抗可以抑制 TCS 对 OVA 诱导的特异性 IgE 的形成, 但重组 IL-4 本身并不能引起 OVA 特异的 IgE 应答反应. 在 TCS 的初次和二次免疫中均有较高的表达峰, 且 CD40L 的表达峰与 IL-4 类似, 仅持续较短的时间. **结论:** IL-4 对于 TCS 诱导的 IgE 应答反应为必要非充分条件. CD40L、TNF- α 和 IL-13 可能也参与了此过程, 其中 CD40L 可能具有与 IL-4 同样重要的作用.

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